



Phytochemical Screening, Cytotoxicity, and Antioxidant Activities of Leaves Extracts from *Eucalyptus citriodora*

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Abstract

The genus *Eucalyptus* has been used in African traditional medicine for the treatment of cardiovascular diseases and diabetes. In this work, chloroform and methanol extracts from the leaves of *Eucalyptus citriodora* was investigated for their phytochemicals, cytotoxicity and antioxidant potentials. Phytochemical screening of the extracts showed the presence of alkaloids, flavonoids, phenols, reducing sugar and steroids in the methanol extract, while the chloroform extract presence of these phytochemicals was observed with exception of reducing sugar. IR absorptions of the extract supported the presence of these phytochemicals by revealing the bands 3335 cm^{-1} (O-H), 1613, 1480 cm^{-1} (C=C) and 1721 cm^{-1} (C=O). Evaluation of the cytotoxicity of the methanol extract using brine shrimp assay, suggested that, the extract was not toxic with LC_{50} value of 1.64 mg/mL. Antioxidant potentials of both chloroform and methanol extracts were determined using phenolic content quantification and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. Methanol extract (37.32 μg TAE/mg) had higher phenolic content than the chloroform extract (12.09 μg TAE/mg). The radical scavenging potentials of the extracts recorded percent inhibitions (1000 $\mu\text{g/mL}$) of 51.33%, 93.05%, 94.43% and 97.80% for chloroform extract, methanol extract, butylated hydroxytoluene and ascorbic acid, respectively. These results showed that *E. citriodora* might contain promising antioxidant agents.

Keywords: Eucalyptus, phytochemicals, brine shrimp, antioxidant

1. Introduction

Medicinal plants are considerably useful and economically essential, they contain active constituents that are used in the treatment of many human diseases [1]. Plants are considered one of the most promising and important subjects that should be explored for the discovery and development of newer and safer drug candidates [2]. Antioxidants play an important role in neutralizing free radical species which are produced as end or by-products of normal biochemical reactions in normal system [3]. Free radicals are highly energetic unstable reactive species containing odd electrons that can penetrate cells and tissues that led to abnormal cell growth (mutation). Moreover, the high accumulation rate of such harmful species in a living body is known as oxidative stress that resulted as a starting point of cancer disease [4].

The lethality to brine shrimp is recommended as an effective pre-screening for cytotoxicity and antitumor assays. A number of studies have established the use of the brine shrimp assay to screen plants commonly used as pesticides, anticancer, and with molluscicidal, larvicidal, fungicidal, and cytotoxic activity [5, 6, 7, 8].

Eucalyptus citriodora is a tall tree with height varying between 20 and 40m. Its leaves are alternate, hanging to limb in the shape of forgery, green coloured and a strong characteristic odour. The leaves of *E. citriodora* have been used to treat several pathologies of which among others: the obesity, the ageing, the cardiovascular illnesses and the diabetes [9]. Essential oils from eucalyptus species were also widely used for cleansing air, in modern cosmetics and room fresheners, food, and pharmaceutical industries [10]. This work aimed to assess phytochemicals present in chloroform and methanol extracts from the leaves of *E. citriodora* as well as their cytotoxic and antioxidant activities.

2. Experimental section

2.1. Plant materials

The leaves of *Eucalyptus citriodora* were collected from Bayero University old campus on the 30th day of November 2014. A voucher specimen (BUKHAN 0028) was deposited in the herbarium of the Department of Plant Biology, Bayero University, Kano, Nigeria.

2.2. Preparation of plant extracts

The freshly collected leaves were rinsed with tap water followed by distilled water to remove the dirt on the surface and allowed to dry. The dried leaves of *E. citriodora* (75.0 g) were ground and cold extracted successively with chloroform (500 mL) and methanol (500 mL). The samples were concentrated using rotary evaporator to give the respective crude extracts (Table 1). The percentage recovery of a crude extract was determined based on the dried weight [11].

2.3. Phytochemical screening

Qualitative tests were carried out in the chloroform and methanol extracts using standard procedure to identify various secondary metabolites [12, 13].

2.4. Thin Layer Chromatography (TLC) of the Extracts

The TLC was performed by spotting the sample using capillary tube on precoated aluminum silica gel plate (4×5 cm). Each plate was developed in suitable solvent system, allowed to dry. The plates were visualized under UV shorter and longer wave length (254 nm and 365 nm) and also stained with iodine [14].

2.5. Infrared Spectroscopic Analysis of the Extracts

Infrared (IR) absorptions of the extracts were measured on ATR-FTIR spectrophotometer using attenuated total reflectance (ATR) technique. The

crystal area was cleaned up and the background was collected. The extract is then placed onto the small crystal area for the IR measurement [15].

2.6. Determination of Total Phenolic Content

The total phenolic contents in the plant extracts were determined using method of Singleton et al [16] with a slight modification. The plant extract (1 mg/ml, 20 µl) was mixed with of folin–ciocalteau reagent (20 µl) in a 96 well micro-titter plate. After 5 minutes, sodium carbonate (0.01M, 20 µl) was added to each sample and allowed to stand for 5 minutes before adding 125 µl of distilled water. The mixture was measured at the absorbance of 765 nm using Thermo-scientific multiskan GO spectrophotometer (Thermofisher Scientific, Vartaa, Finland). The blank is the same reaction mixture with water instead of the extracts or standard. The total phenolic content was expressed as µg TAE/mg mg gallic acid equivalent per gram.

2.7. 2,2-Diphenyl-2-picrylhydrazyl (DPPH) Radical-Scavenging Assay

The free radical scavenging activity of the plant extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined according to the method described in [17] with slight modification. Each sample of stock solution (1.0 mg/L) was diluted to final concentrations of 1000, 500, 250, 125, 62.5, 31.3 and 15.63 µg/mL. Then, a total of 50 µM DPPH methanolic solution (160 µL) was added to sample solution (40 µL) and allowed to react at room temperature for 30 minutes in dark. The absorbance of the mixtures was measured at 517 nm. Ascorbic acid was used as positive control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. Inhibitions of DPPH radical in percent (I%) were calculated using the equation 1.

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100 \quad (\text{Equation 1})$$

Where:

A_{blank} is the absorbance value of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance values of the test compounds.

The sample concentration that provides 50% inhibition (IC₅₀) was determined using SPSS 16.

2.8. Brine Shrimp Lethality Test

Brine shrimp eggs, *Artemia salina* (*A. salina*) were hatched in artificial seawater prepared by dissolving 19 g of sea salt in 500 mL of distilled water. After 48 h incubation at room temperature (22-29 °C), the larvae was attracted to one side of the vessel with a light source and collected by pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater [18].

2.9. Brine shrimp assay

Toxicity of the extract was monitored by the brine shrimp lethality test according to a standard method [18]. Each of the extract (1 mg/mL) was dissolved in methanol, from which 5 000, 500 and 50 µL of each solution was transferred into vials corresponding to 1.00, 0.10 and 0.01 mg/mL respectively. This was allowed to evaporate to dryness in about 24 h at room temperature. Each dosage was tested in triplicate (9 per test sample). Sea water (4 mL) and 10 larvae were introduced into each vial. The final volume of solution in each vial was adjusted to 5 mL with seawater immediately after adding the shrimps. A negative control was prepared as a drug-free. Survivors were counted after 24 h, and LC₅₀ was determined by probit analysis using SPSS version 16.

3. Results and Discussion

Dried powdered leaves of *E. citriodora* were subjected to solvent extraction using chloroform and methanol in the polarity gradient system. Methanol extract (5.6%) was observed to be higher than chloroform extract (4.0%) as presented in table 1. According to Dapkevicius et al. [19] the amount of material extracted using different solvents could be associated with composition of each particular herb, differences in the solubility of extractives and their polarity as well as extraction technique employed. The methanol extract in this work demonstrated that, polar constituents are relatively higher than the medium polar constituents from the leaves of the plant.

Table 1: Appearance of *E. citriodora* extracts

Extract	Weight (g)	Yield (%)	Appearance
Chloroform extract (ECCH)	3.0	4.0	Green, Gummy
Methanol extract (ECME)	4.2	5.6	Green, Gummy

$$\text{Yield (\%)} = \frac{\text{Mass of extract}}{\text{Mass of plant material}} \times 100 \quad (\text{Equation 2})$$

Qualitative tests were carried out on extracts of *E. citriodora* to investigate the presence of some classes of secondary metabolites (table 2). The extracts showed the presence of alkaloids, flavonoids, phenols and steroids. Reducing sugar was observed in methanol extract only. These secondary metabolites have been reported to demonstrate anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral and many other activities to a greater or lesser extent [20]

Table 2: Phytochemical analysis of *E. citriodora*

Sample	Phytochemical tested				
	Alkaloids	Flavonoids	Phenols	Reducing sugar	Steroids
ECCH	+	+	+	-	+
ECME	+	+	+	+	+

ECCH= chloroform extract; ECME= methanol extract; + = present; - = absent.

Kodorou *et al.*, [221] also reported the presence of these phytochemicals and also the quinones, coumarins, saponosides and anthocyanins from the ethanol extract of *E. citriodora* leaves. The wealth of these secondary metabolites could explain the use of the plant in the threshold of traditional and ethnomedicine.

Thin-layer chromatographic analysis of the extracts of *E. citriodora* was conducted on a precoated silica-gel plate using two different

solvent systems comprising of only petroleum ether and chloroform. The number of spots and retention factors (R_f) of the extracts are presented in table 3. All the extracts gave a single spot with the solvent combinations of petroleum ether-chloroform (4:1 and 3:2) for the respective R_f value.

Table 3: TLC profile of *E. citriodora* extracts

Extract	Spot(s)	R_f	Solvent system PE:CH
ECCH	1	0.23	4:1
	1	0.18	3:2
ECME	1	0.21	4:1
	1	0.15	3:2

ECCH= chloroform extract; ECME= methanol extract; PE = petroleum ether; CH = chloroform

$$\text{Retention factor} = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent front}} \quad (\text{Equation 3})$$

Infrared spectroscopic analysis of extracts from the leaves of *E. citriodora* was performed using an ATR-FTIR spectrometer to assess possible functional groups present in both extracts. Functional groups that might be associated with steroids, flavonoids and phenols were observed in the IR absorptions 3335 cm^{-1} (O-H), 2918 cm^{-1} (C-H), 1689 cm^{-1} (C=O) presented in Figure 1 and Table 4 for chloroform extract (ECCH). While the ECME, exhibited absorptions due to carboxylic acid at 3335 cm^{-1} and aldehydic carbonyl at 1721 cm^{-1} (Table 4).

The total phenolic content (TPC) of the plant extracts was determined using the Folin-Ciocalteu reagent expressed as tannic acid equivalence ($\mu\text{g TAE/mg}$) and gallic acid equivalence ($\mu\text{g GAE/mg}$) from standard linear regression equations $y = 0.0009x + 0.301$ ($R^2 = 0.96$) and $y = 0.0031x + 0.4566$ ($R^2 = 0.88$), respectively.

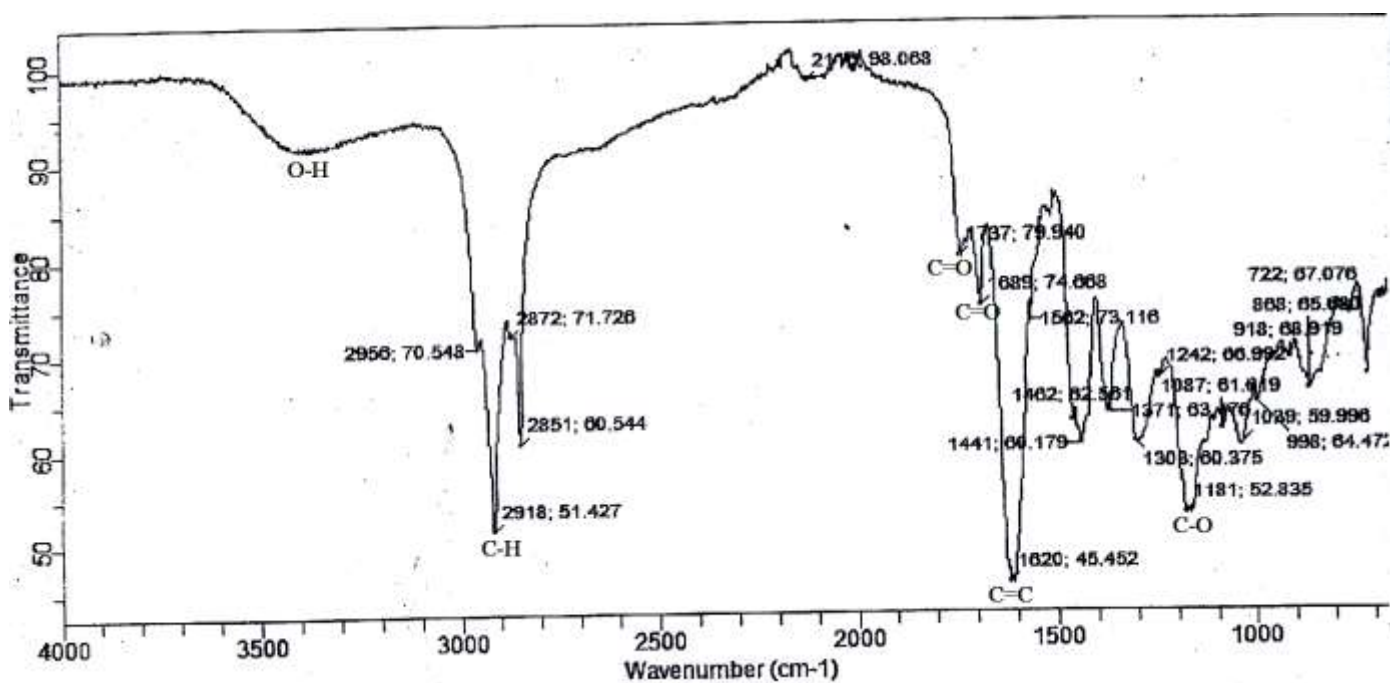


Figure 1: IR spectrum of ECCH

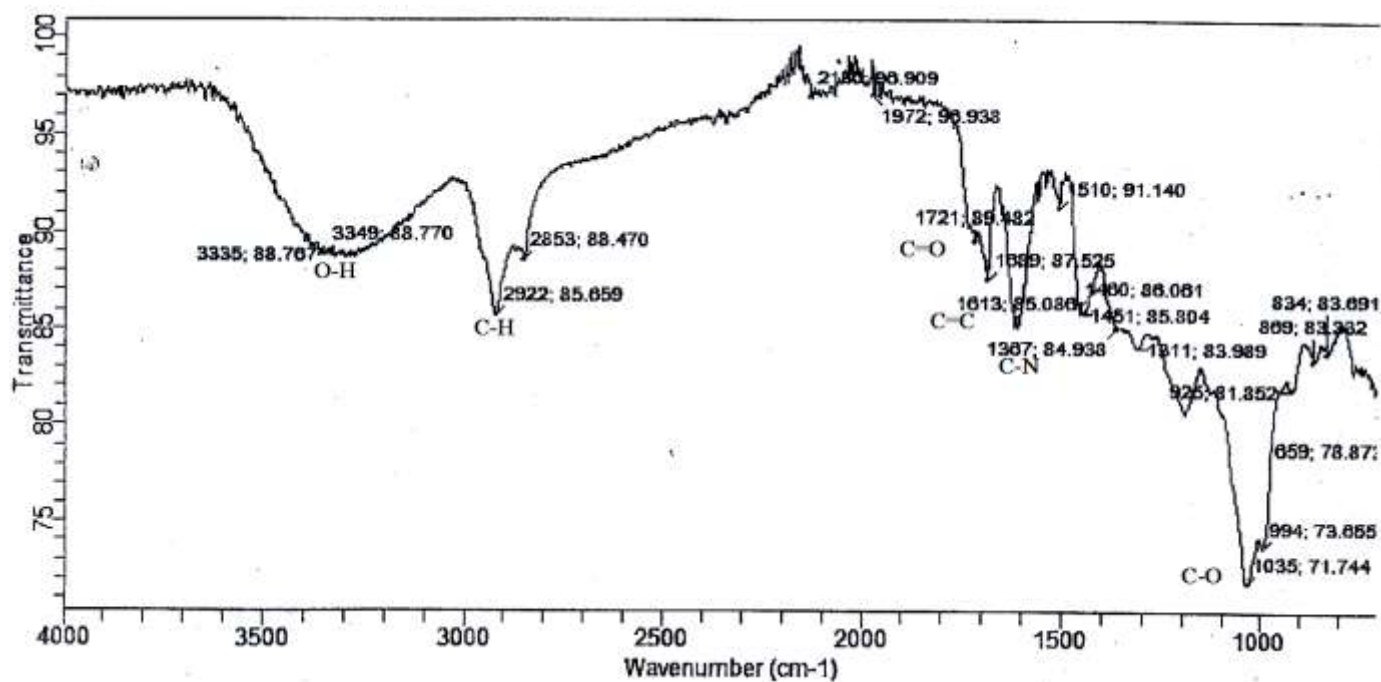


Figure 2: IR spectrum of ECME

Table 4: FTIR analysis of *E. citriodora* extracts

Sample	Absorption bands (cm ⁻¹)	Bond	Functional Group
ECCH	3400	O-H	Alcohols
	2918, 2956	C-H stretch	Alkanes
	2851, 2872	H-C=O, C-H stretch	Aldehydes
	1737	C=O stretch	Esters
	1689	C=O stretch	Amides
	1620	C=C stretch	Alkenes
	1441,1462,1562	C=C stretch	Aromatics
	1181	C-O stretch	Alcohols
ECME	3335, 3349	O-H stretch	Carboxylic acids, Alcohols
	2922	C-H stretch	Alkanes
	2853	H-C=O, C-H stretch	Aldehydes
	1721	C=O stretch	Aldehyde
	1613	C=C	Alkenes
	1035	C-O	Alcohols, ethers

The technique used to obtain a crude estimate of the amount of phenolic compounds present in an extract *via* complex redox reaction with phosphotungstic-phosphomolybdic acids in alkaline solution to form a blue coloured complex in the presence of phenolics (Table 5). High phenolic content was exhibited by methanol extract as compared with tannic and gallic acids at concentrations of 37.3 µg TAE/mg and 58.1 µg GAE/mg, respectively. Generally, extracts that contain a high amount of polyphenols also exhibit high antioxidant activity [22].

Table 5: Phenolic Content of *E. citriodora* extracts

Extract	Phenolic content (µg TAE/mg)	Phenolic content (µg GAE/mg)
ECCH	12.1	-
ECME	37.3	58.1

ECCH = chloroform extract; ECME = methanol extract; GAE = Gallic acid equivalence and TAE = tannic acid equivalence

The stable radical DPPH has been used widely for the assessment of primary antioxidant activity, that is, the free radical scavenging

activities of an antioxidant compounds, plant and fruit extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 515 nm [22].

The radical scavenging at 1000 µg/mL of methanol and chloroform extracts were exhibited at 93.05% and 51.33%, respectively. Positive controls butylatedhydroxytoluene (94.43%) and ascorbic acid (97.80%) had a comparative antioxidant capacity to that of methanol extract. A similar trend in inhibition of DPPH radical was reported by Kodorou *et al.*, [21].

The brine shrimp lethality test was carried in order to determine the lethality of methanol extracts of *E. citriodora* against the brine shrimp larvae. The percentage mortality at different concentrations as well as the LC₅₀ value determined by probit analysis using SPSS version 16 are shown in table 6. Methanol extracts of *E. citriodora* was found toxic to brine shrimp larvicidal activity at the concentration of 1.65 mg/mL. This was based on Meyer *et al.*, [23], that a drug is toxic (active) if it has an LC₅₀ value of less than 1mg/mL while non-toxic (inactive) if it is greater than 1mg/mL.

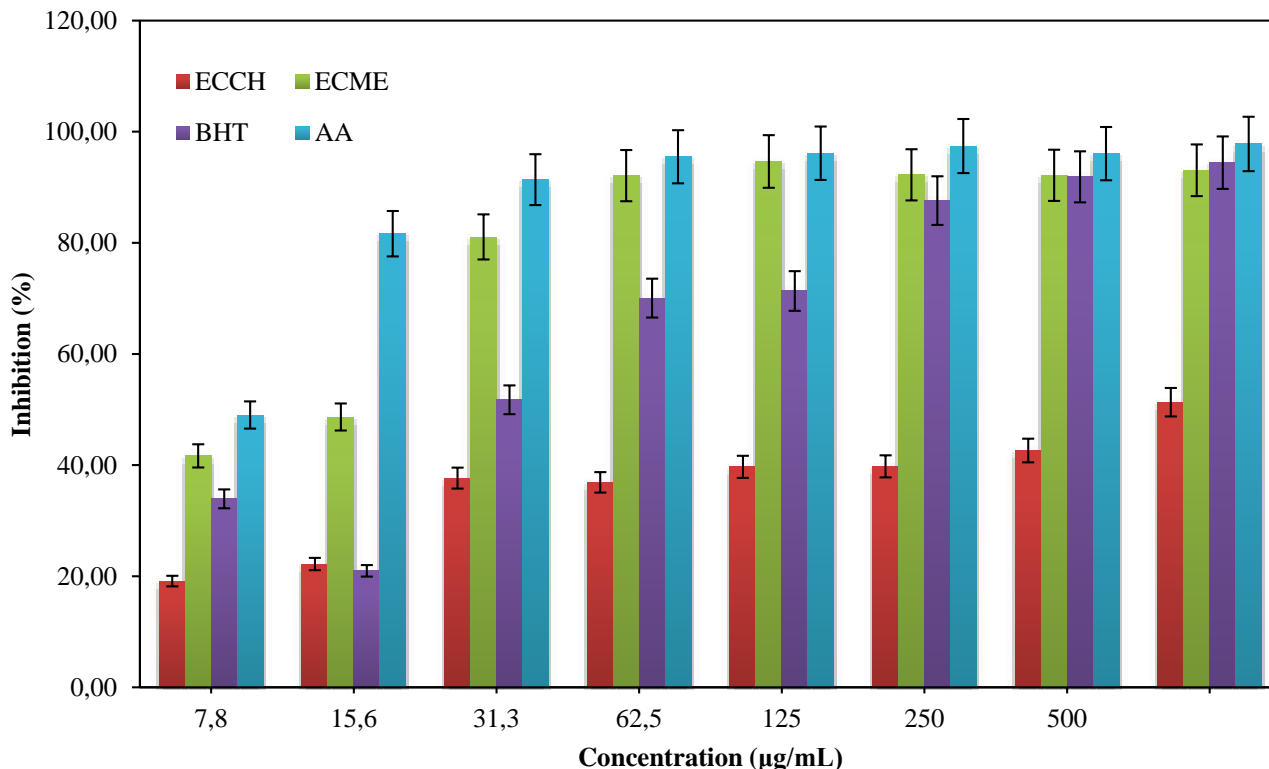
Figure 3. Antioxidant activity of *E. citriodora* extracts and positive controls (Butylated hydroxytoluene and ascorbic acid)

Table 6. Brine shrimp toxicity of methanol extract (ECME)

Conc. (mg/ml)	No. of surviving larvae			Total no. of surviving	% Mortality (%M)	LC ₅₀ (mg/ml)
	T1	T2	T3			
1	0	0	0	0	100	1.649
0.1	0	2	2	4	87	
0.01	1	3	1	5	83	

4. Conclusion

E. citriodora extracts had demonstrated a significant antioxidant and cytotoxic properties that could be connected with the phytochemicals presence. Functional groups that might be associated with steroids, flavonoids and phenols were observed in the IR absorptions 3335 cm^{-1} (O-H), 2918 cm^{-1} (C-H), 1689 cm^{-1} (C=O). Therefore results of this study indicate that, the chloroform and methanol extracts are rich in various bioactive compounds and may find use in pharmaceuticals and other applications.

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